Combination therapy with AT1 blocker and vitamin D analog markedly ameliorates diabetic nephropathy: Blockade of compensatory renin increase

Zhongyi Zhang*, Yan Zhang*, Gang Ning†, Dilip K. Deb*, Juan Kong*, and Yan Chun Li*‡

*Department of Medicine, Division of Biological Sciences, University of Chicago, Chicago, IL 60637; and †The Huck Institutes of the Life Sciences, Pennsylvania State University, University Park, PA 16802

Edited by John T. Potts, Jr., Massachusetts General Hospital, Charlestown, MA, and approved August 12, 2008 (received for review April 17, 2008)

The renin–angiotensin system (RAS) plays a critical role in the development of diabetic nephropathy, and blockade of the RAS is currently used for treatment of diabetic nephropathy. One major problem for the current RAS inhibitors is the compensatory renin increase, which reduces the efficacy of RAS inhibition. We have shown that vitamin D exerts renoprotective actions by transcriptionally suppressing renin. Here we demonstrated that combination therapy with an AT1 receptor blocker and a vitamin D analog markedly ameliorated renal injury in the streptozotocin (STZ)-induced diabetes model due to the blockade of the compensatory renin rise by the vitamin D analog, leading to more effective RAS inhibition. STZ-treated diabetic DBA/2J mice developed progressive albuminuria and glomerulosclerosis within 13 weeks, accompanied by increased intrarenal production of angiotensin (Ang) II, fibronection, TGF-β, and MCP-1 and decreased expression of slit diaphragm proteins. Treatment of the diabetic mice with losartan or paricalcitol (19-nor-1,25-dihydroxyvitamin D_2, an activated vitamin D analog) alone moderately ameliorated kidney injury; however, combined treatment with losartan and paricalcitol completely prevented albuminuria, restored glomerular filtration barrier structure, and markedly reduced glomerulosclerosis. The combined treatment suppressed the induction of fibronection, TGF-β, and MCP-1 and reversed the decline of slit diaphragm proteins nephrin, Neph-1, ZO-1, and α-actinin-4. These were accompanied by blockade of intrarenal renin and Ang II accumulation induced by hyperglycemia and losartan. These data demonstrate that inhibition of the RAS with combination of vitamin D analogs and RAS inhibitors effectively prevents renal injury in diabetic nephropathy.

albuminuria | glomerulosclerosis | renin–angiotensin system

Diabetic nephropathy is the most common renal complication that often leads to end-stage kidney disease and high mortality (1). Previous studies have suggested the renin–angiotensin system (RAS) as a major mediator of progressive renal injury in diabetic nephropathy. Drugs targeting the RAS including angiotensin (Ang)-converting enzyme inhibitors (ACEIs) and Ang II type 1 receptor blockers (ARBs) have been shown to reduce the progression of glomerulosclerosis, tubulointerstitial fibrosis, and proteinuria (2–6). The systemic components of the RAS are actually down-regulated in diabetic mellitus (7), whereas renal interstitial Ang II levels are estimated to be 1,000-fold higher than in the plasma (8); therefore, intrarenal RAS is thought to play the major damaging role. In fact, all components of the RAS are present within the kidney (9). Cells in the kidney are able to synthesize renin, prorenin/renin receptor (10), angiotensinogen, and Ang II receptors independent of the systemic RAS (11), making the kidney capable of maintaining a high level of intrarenal Ang II. Intrarenal renin and angiotensinogen levels are induced in diabetic animals (12, 13), and high glucose has been shown to stimulate renin and Ang II synthesis in mesangial cells and podocytes (14–16). Intrarenal Ang II exerts multiple effects on the kidney that promote the progression of renal injury; these include an increase in glomerular capillary pressure, induction of profibrotic and proinflammatory cytokine production, promotion of immune cell infiltration, stimulation of cell proliferation and hypertrophy, up-regulation of extracellular matrix (ECM) synthesis, and damaging of podocytes (17–19).

1,25-Dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], the hormonal form of vitamin D, is a negative endocrine regulator of the RAS (20). $1,25(OH)_2D_3$ suppresses renin biosynthesis (21), and null mutant mice lacking the vitamin D receptor (VDR) gene develop hyperreninemia, high blood pressure, and cardiac hypertrophy (22–24). Our recent studies showed that in diabetic state VDR-null mice developed more severe nephropathy than wild-type mice (25), suggesting that vitamin D plays a protective role against hyperglycemia-induced renal injury by regulation of the RAS. However, whether vitamin D or vitamin D analogs have therapeutic effects in intervention or prevention of diabetic nephropathy remains to be tested.

Although RAS inhibitors, including ACEIs and ARBs, are widely used in the therapy of renal and cardiovascular diseases, the major problem of these drugs is the compensatory renin rise due to the disruption of the feedback inhibition of renin production (26). The increase in renin activity stimulates the conversion of Ang I and ultimately Ang II, which largely limits the efficacy of RAS inhibition (27, 28). The increased renin can also act through the prorenin/renin receptor (10), which may cause renal and cardiovascular damages independent of Ang II (29). Given that low-calcemic vitamin D analogs are able to inhibit renin expression in animals (30, 31), we reasoned that combining vitamin D analogs with RAS inhibitors to suppress the reactive renin increase should generate better therapeutic effects (32). To test this concept, in the current study we treated a mouse model of diabetic nephropathy with a combination of losartan and paricalcitol. Paricalcitol (19-nor-1,25-dihydroxyvitamin D_2) is an activated vitamin D analog and activates VDR with a high affinity, and we used paricalcitol because it suppresses renin expression in mice with the same potency as calcitriol but without induction of hypercalcemia (31). Our data demonstrated that the combination of an ARB and a vitamin D analog markedly ameliorated renal injury because of more effective inhibition of the RAS within the kidney.

Results

We selected DBA/2J mice in this study because this strain has been shown to be more susceptible to hyperglycemia-induced renal

Author contributions: Z.Z. and Y.Z. contributed equally to this work; Z.Z., Y.Z., and Y.C.L. designed research; Z.Z., Y.Z., and G.N. performed research; D.K.D. and J.K. contributed new reagents/analytical tools; Y.Z. and Y.C.L. analyzed data; and Y.C.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

‡To whom correspondence should be addressed at: Department of Medicine, University of Chicago, MC 4076, 5841 South Maryland Avenue, Chicago, IL 60637. E-mail: cyan@medicine.bsd.uchicago.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0803751105/DCSupplemental.

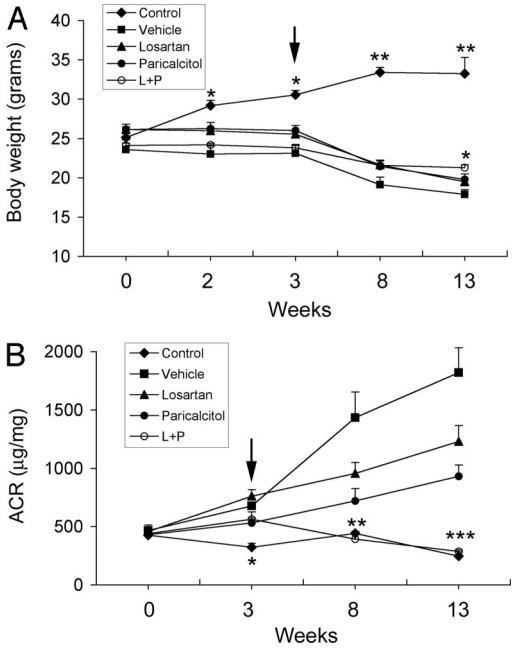

Fig. 1. Effect of the drug treatments on body weight and development of albuminuria in STZ-induced diabetic mice. Eight-week-old DBA/2J mice were injected i.p. with 40 mg/kg STZ for five consecutive days in the first week (week 0). Two weeks after STZ injection (week 3), the mice were treated with vehicle (V), losartan (L, 0.025 mg/ml), paricalcitol (P, 0.4 μg/kg), or losartan and paricalcitol (L+P), and their body weight and urinary albumin levels were monitored for up to 13 weeks. Control (C) mice are nondiabetic mice without STZ injection or any treatment. (*A*) Body weight curves. *, $P < 0.05$; **, $P < 0.01$ (vs. V). (*B*) Urinary ACR. Note the dramatic inhibition of albuminuria in the L+P group. The arrows indicate the start of the drug treatment. *, $P < 0.05$ (C vs. other groups); **, $P < 0.01$ (C and L+P vs. V); ***, $P < 0.001$ (C and L+P vs. V). $n = 4–6$.

injury compared with other strains in the streptozotocin (STZ)-induced diabetes model (33, 34). Two weeks after STZ treatment we randomized the mice into four groups and treated them respectively with vehicle, losartan (0.025 mg/ml), paricalcitol (0.4 μg/kg), or a losartan and paricalcitol combination (L+P) for 10 weeks, with nondiabetic mice receiving no treatment as the negative control. Blood glucose levels in all diabetic groups rose to >500 mg/dl 2 weeks after STZ treatment; the high glucose levels persisted during the 13-week period and were not significantly affected by the drug treatments [supporting information (SI) Table S1]. As expected, in contrast to the nondiabetic mice, all STZ-treated diabetic mice failed to gain weight during the 13 weeks; however, L+P treatment appeared to maintain the mouse body weight significantly better than other treatments (Fig. 1*A*). As shown in Fig. 1*B*, the vehicle-treated diabetic mice developed time-dependent, progressive albuminuria, with the urinary albumin-to-creatinine ratio (ACR) increased almost 4-fold at week 13. Losartan or paricalcitol treatment moderately ameliorated the development of albuminuria. Most striking, however, is the complete prevention of albuminuria by L+P treatment; the ACR was basically indistinguishable from the negative control after 5 weeks or 10 weeks of L+P treatment (Fig. 1*B*). Consistently, plasma creatinine levels were elevated in vehicle-treated mice, which were significantly reduced by L+P treatment (Fig. S1). Therefore, combination therapy with an ARB and a vitamin D analog markedly prevents the development of proteinuria in diabetic animals.

Podocyte injury plays a key role in the development of albuminuria in diabetic nephropathy (17). To explore the mechanism underlying the prevention of proteinuria, we examined the structure of glomerular filtration barrier by electron microscopy. As shown in Fig. 2, normal morphology of the glomerular basement membrane (GBM) and podocyte foot processes were seen in the nondiabetic control mice (Fig. 2*A*). As expected, chronic hyperglycemia caused a marked increase in GBM thickness and severe foot process effacement in vehicle-treated mice (Fig. 2 *B* and *F*), consistent with the dramatic albuminuria observed in these mice (Fig. 1*B*). Although both losartan and paricalcitol treatment moderately reduced the increase of GBM thickness and foot process effacement (Fig. 2 *C*, *D*, and *F*), it was the L+P treatment that basically prevented the thickening of GBM and the development of effacement (Fig. 2 *E* and *F*).

The slit diaphragm is the key structure that controls protein filtration through the glomerular filtration barrier (35). We showed previously that decrease of nephrin, a key structural protein of the slit diaphragm, was associated with the severe proteinuria in diabetic VDR-null mice, whereas podocin, another slit diaphragm protein, was not affected in the mutant mice (25). We therefore

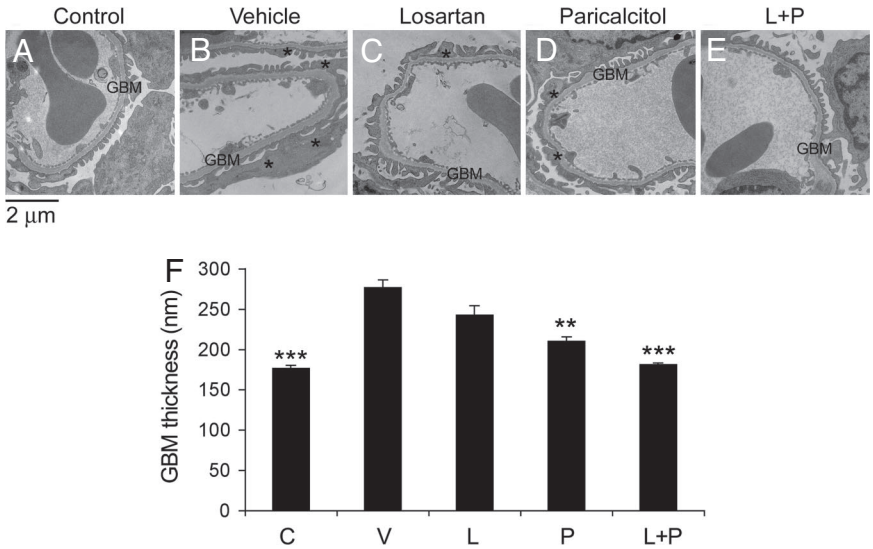

Fig. 2. Transmission electron microscopy of the GBM. Kidney samples from nondiabetic control mice (*A*) and diabetic mice treated with vehicle (*B*), losartan (*C*), paricalcitol (*D*), and L+P (*E*) were subject to electron microscopic analyses. Stars indicate the areas of podocyte foot process effacement. (*F*) Quantification of the thickness of the GBM from these groups as indicated. **, $P < 0.01$; ***, $P < 0.001$ (vs. V).

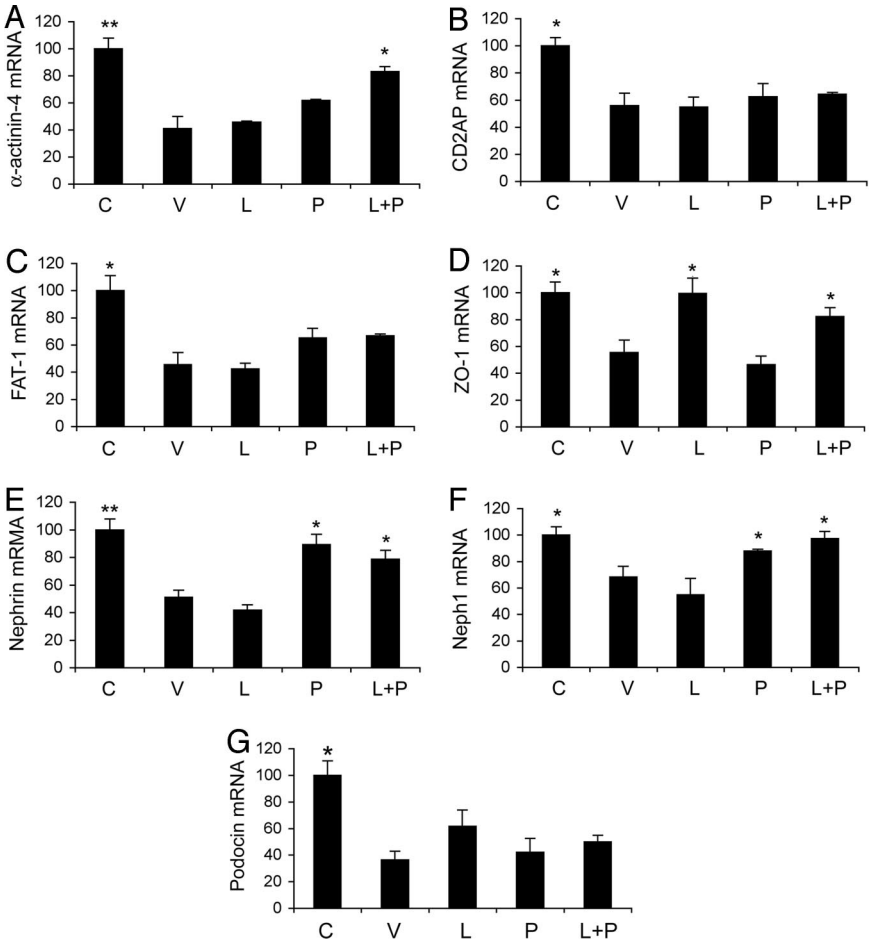

Fig. 3. Expression of slit diaphragm proteins. Total RNAs were extracted from kidneys of nondiabetic control mice (C) and diabetic mice treated with vehicle (V), losartan (L), paricalcitol (P), or L+P at 13 weeks. The mRNA levels of α-actinin-4 (*A*), CD2AP (*B*), FAT-1 (*C*), ZO-1 (*D*), nephrin (*E*), Neph-1 (*F*), and podocin (*G*) were determined by real-time RT-PCR. *, $P < 0.05$; **, $P < 0.01$ (vs. V). $n = 3–4$.

quantified the expression of the key structural proteins forming the slit diaphragm by real-time RT-PCR. As shown in Fig. 3, compared with the nondiabetic control, the mRNA levels of α-actinin-4, CD2AP, FAT-1, ZO-1, nephrin, Neph-1, and podocin were all reduced in vehicle-treated diabetic mice (Fig. 3). All drug treatments had no effect on the decline of CD2AP, FAT-1, and podocin; however, L+P treatment almost restored the expression of α-actinin-4, ZO-1, nephrin, and Neph-1 (Fig. 3). The changes in nephrin and podocin are consistent with our early observations in VDR-null mice (25).

Histological examination of the kidney revealed increased glomerulosclerosis in vehicle-treated diabetic mice (Fig. 4*B*) relative to nondiabetic mice (Fig. 4*A*) at week 13, with increased ECM accumulation in the mesangium. Losartan (Fig. 4*C*), paricalcitol (Fig. 4*D*), or L+P (Fig. 4*E*) all reduced glomerular sclerosis. Semiquantitative scoring of glomerulosclerosis in the kidney sections confirmed the highest score in vehicle-treated mice and the significant reduction of the score in L+P-treated mice (Fig. 4*K*). Consistently, the expression of fibronectin (FN), one of the major ECM proteins, was markedly induced in vehicle-treated mice (Fig. 4*G*) compared with the nondiabetic control (Fig. 4*F*), as determined by immunostaining (Fig. 4 *F–J*), quantitative PCR (Fig. 4*L*), and Western blotting (Fig. 4 *M* and *N*); the drug treatments, particularly L+P treatment (Fig. 4*J*), mostly prevented the induction of FN at both the mRNA and protein levels in the diabetic mice (Fig. 4 *L–N*).

TGF-β plays a major role in the development of glomerulosclerosis, and MCP-1 recruits monocytes/macrophages into the kidney to initiate inflammation that leads to renal injury. As expected, compared with control mice, vehicle-treated diabetic mice showed elevation of TGF-β and MCP-1 in the kidney, and the induction of these factors was almost completely suppressed by L+P treatment (Fig. S2), consistent with the improved glomerular and proteinuric phenotypes in the cotreated mice.

It is well known that hyperglycemia induces the intrarenal RAS, leading to accumulation of Ang II in the kidney and causing renal injury (16, 19, 25, 36). To determine whether L+P treatment more effectively suppressed the activation of the RAS, we determined intrarenal Ang II levels at week 13. Immunostaining showed that hyperglycemia markedly induced Ang II accumulation within the glomerular and renal tubular regions (Fig. 5 *B* and *G*); as expected, losartan treatment further enhanced the Ang II levels in both regions (Fig. 5 *C* and *H*) because of the compensatory increase of renin (Fig. 5 *M* and *N*). These observations were confirmed by Western blot analysis of the kidney lysates (Fig. 5 *K* and *L*). Interestingly, L+P treatment basically eliminated the accumulation of Ang II induced by hyperglycemia or by losartan treatment, revealed by immunostaining (Fig. 5 *E* and *J*) as well as by Western blotting (Fig. 5 *K* and *L*). Consistently, and as expected, Western blotting and quantitative PCR showed that paricalcitol drastically suppressed renin expression in the kidney (Fig. 5*N*), whereas L+P treatment markedly attenuated the renin increase induced by losartan at both the protein (Fig. 5*M*) and mRNA (Fig. 5*N*,

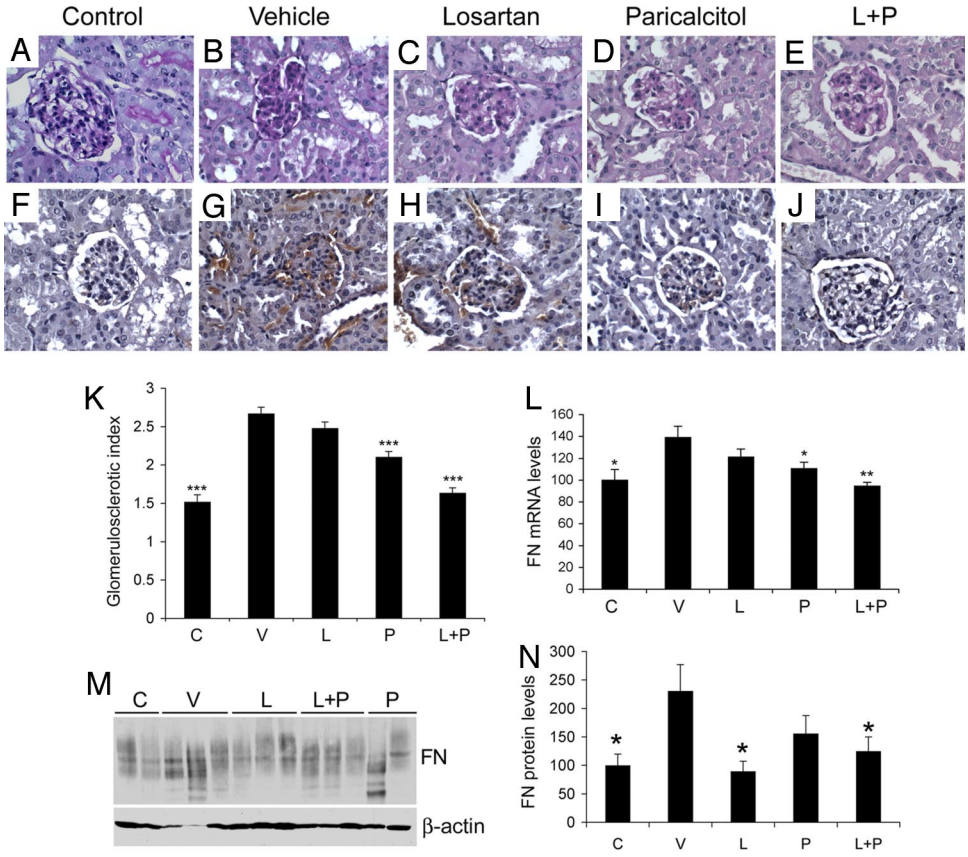

Fig. 4. Effect of the treatments on the development of glomerulosclerosis. Nondiabetic control and the four treatment groups of diabetic mice were killed 13 weeks after STZ treatment, and the kidneys were harvested for histological and immunohistological analyses. (*A–E*) Representative glomerular morphology of nondiabetic control (*A*) and diabetic mice treated with vehicle (*B*), losartan (*C*), paricalcitol (*D*), or L+P (*E*). The sections were stained with periodic acid-Schiff (PAS). (*F–J*) Fibronectin expression determined by immunostaining. Representative glomerular sections from nondiabetic control (*F*) and diabetic mice treated with vehicle (*G*), losartan (*H*), paricalcitol (*I*), or L+P (*J*) were immunostained with anti-fibronectin antibody. (*K*) Semiquantitative glomerulosclerotic scores of nondiabetic control (C) and diabetic mice treated with vehicle (V), losartan (L), paricalcitol (P), or L+P. (*L*) Real-time RT-PCR quantification of fibronectin mRNA levels in the kidney of all treatment groups as indicated. (*M*) Western blot analyses of fibronectin protein levels in kidney lysates from all treatment groups as indicated. (*N*) Densitometric quantification of fibronectin protein levels based on Western blot data. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (vs. V).

compare L and L+P) levels. The inhibition of renin increase and Ang II accumulation in the kidney is most likely the basis for the effective prevention of renal injury by the combination therapy.

Discussion

Diabetic nephropathy is a long-term complication of diabetic mellitus, and effective blockade of the progression of nephropathy remains a medical challenge. Because activation of the RAS in the kidney is a major mediator of renal injuries in diabetic nephropathy (12, 19), small-molecule inhibitors targeting the RAS (namely ACEIs and ARBs) are currently used for treatment of diabetic nephropathy (37). This therapy is based on the theory that blocking the RAS reduces the intraglomerular pressure and thus proteinuria; however, inhibition of the RAS also ameliorates blood pressure-independent renal injury caused by Ang II (38). The STZ-induced diabetic model is not a hypertensive model; therefore, the therapeutic effects seen in this study are blood pressure-independent. The efficacy of the RAS-targeting drugs is often compromised by the reactive renin increase caused by disruption of the renin feedback inhibition (26, 32). This clinical problem is not solved even with the use of the new renin inhibitor aliskiren (39), which blocks the enzymatic activity but not the production of renin. Patients treated with aliskiren had plasma immunoreactive renin increased to a level higher than that when valsartan (an ARB) was used (39). High renin buildup increases the risk of Ang II-dependent and -independent organ damages. The finding that $1,25(OH)_2D_3$ represses renin gene transcription (21, 24) provides a good basis to use vitamin D analogs for the suppression of the compensatory renin increase because the analogs directly inhibit renin biosynthesis. Based on this principle, here we demonstrated that combination therapy with an ARB and a vitamin D analog very effectively blocks the development of diabetic nephropathy in a type 1 diabetes mellitus animal model as a result of effective inhibition of renin and Ang II production within the kidney. This finding has important implications for new therapeutic intervention of diabetic nephropathy.

Proteinuria and glomerulosclerosis are the pathological hallmarks of diabetic nephropathy (40). The most profound effect of the combination therapy is the complete prevention of albuminuria in the diabetic mice without a significant change of the blood glucose. Microalbuminuria is a major risk factor for progressive renal function decline in diabetic nephropathy (41) and is thought to be the first step in an inevitable progression to proteinuria and renal failure (42, 43). Thus, reduction of albuminuria is a major target for renoprotective therapy in both type 1 and type 2 diabetes. Clinical studies have demonstrated that ACEIs and ARBs can reduce albuminuria in diabetic patients; however, not all patients respond to the treatment (44), in many cases because of incomplete blockade of the RAS. Vitamin D also has antiproteinuric activity. An early study showed that $1,25(OH)_2D_3$ treatment significantly decreased albuminuria and podocyte hypertrophy in subtotally nephrectomized rats (45). Paricalcitol therapy was also reported to reduce proteinuria in CKD patients (46); however, the mechanism is unclear. In the present study we demonstrated that losartan or paricalcitol alone moderately reduced albuminuria in diabetic mice; interestingly, losartan and paricalcitol appeared to act synergistically, and their combination completely blocked the development of albuminuria. Consistently, the combination therapy completely normalized the structure of the glomerular filtration barrier, preventing GBM thickening and podocyte effacement. At the molecular level, the development of albuminuria was associated with a reduction of the majority of the proteins that form the slit diaphragm. The disturbance in the expression of these proteins likely plays an important role in the pathogenesis of the proteinuric nephropathy. Although losartan or paricalcitol alone can partially restore the expression of some of these proteins, in comparison, their combination restored more proteins (α-actinin-4, ZO-1, nephrin, and Neph-1) to higher levels, consistent with the much better

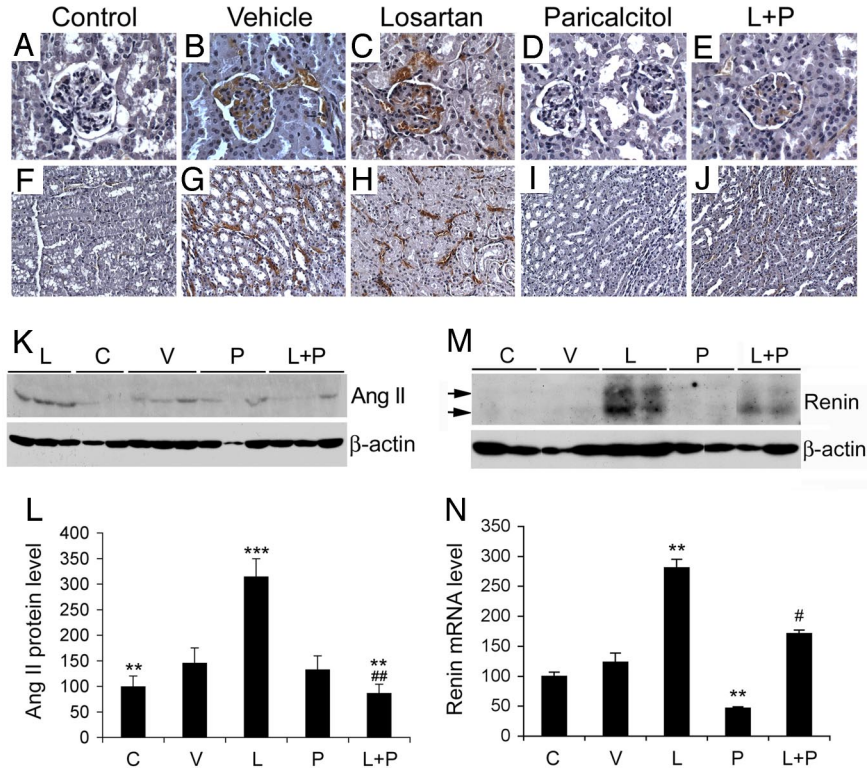

Fig. 5. Effect of the drug treatments on intrarenal Ang II and renin production. (*A–J*) Immunostaining of kidney in glomerular (*A–E*) and tubular (*F–J*) areas with Ang II-specific antibody. Nondiabetic control mice (*A* and *F*) and diabetic mice treated with vehicle (*B* and *G*), losartan (*C* and *H*), paricalcitol (*D* and *I*), or L+P (*E* and *J*) were killed at week 13. (*K*) Western blot analyses of intrarenal Ang II levels in nondiabetic control mice (C) and diabetic mice treated with vehicle (V), losartan (L), paricalcitol (P), or L+P. (*L*) Densitometric quantification of Ang II levels in control and treated group is as indicated. (*M*) Western blot analysis of renin protein levels in kidney lysates from nondiabetic control mice (C) and diabetic mice treated with vehicle (V), losartan (L), paricalcitol (P), or L+P. The arrows indicate the prorenin and renin bands. (*N*) Real-time RT-PCR quantification of renin mRNA levels in the kidneys of these mice. **, $P < 0.01$; ***, $P < 0.001$ (vs. V); #, $P < 0.05$; ##, $P < 0.01$ (vs. L). $n = 3–5$.

antiproteinuric effect. In previous studies, blocking the RAS was shown to normalize nephrin expression (47, 48), and vitamin D can up-regulate nephrin expression in podocytes (49). Together, these data clearly indicate that the podocytes are a major target of the RAS and vitamin D in renoprotection.

The combination of losartan and paricalcitol also reduced glomerulosclerosis in the diabetic mice, which was accompanied by suppression of fibronectin induction at both mRNA and protein levels. The blockade of TGF-β and MCP-1 induction by the combination therapy is in part the upstream molecular events that lead to the reduction of glomerulosclerosis. TGF-β is a major profibrotic factor that plays a key role in glomerulosclerosis in diabetes. Monocyte/macrophage infiltration into the kidney, stimulated by MCP-1 produced from kidney cells in diabetic state, promotes kidney fibrosis and renal injury through secretion of inflammatory cytokines. Although vitamin D alone can directly suppress the high-glucose induction of TGF-β and MCP-1 in mesangial cells (50), this study showed that cotreatment with losartan and paricalcitol achieved better inhibition of these cytokines *in vivo*. Because TGF-β and MCP-1 are up-regulated by Ang II, the suppression of these factors by the cotreatment is likely mediated by the blockade of Ang II accumulation (Fig. 5).

The molecular basis underlying the impressive antiproteinuric and antisclerotic effects of the combination therapy is the blockade of RAS activation within the kidney. Our data demonstrate that the intrarenal accumulation of Ang II was associated with the development of albuminuria and glomerulosclerosis in the vehicle-treated diabetic mice. Ang II promotes nephropathy through multiple pathways, such as stimulating TGF-β and ECM protein production (51), increasing glomerular permeability, down-regulating slit diaphragm proteins, and inducing podocyte injury (17). Consistent with the compensatory renin increase theory, losartan treatment dramatically increased renin as well as Ang II levels in the kidney; however, the induction of renin and Ang II was markedly attenuated in the losartan and paricalcitol cotreatment group (Fig. 5). Therefore, in the combination therapy, paricalcitol blocks the increase in renin and thus Ang II, losartan antagonizes the actions of the already reduced Ang II by blocking the AT1 receptor, and the reduction in renin minimizes the Ang II-independent activation of prorenin/renin receptor (10), which may also cause renal damage (52). Together these actions of the combination therapy lead to an effective prevention of the development of diabetic nephropathy. Given the multiple functionalities of vitamin D, however, the renoprotective effect of paricalcitol may not be limited to the regulation of the RAS (53).

This is the first demonstration that vitamin D analogs can be used to block the compensatory renin increase in a combination therapy with RAS inhibitors. The promising therapeutic effects of the combination seen in diabetic mice provide insight into the pharmacological intervention of diabetic nephropathy. However, given the limitation of animal models, whether this therapeutic strategy also works in humans requires further clinical investigations.

Methods

Animals and Treatment. Eight-week-old male DBA/2J mice (The Jackson Laboratory) were induced to diabetes by i.p. injection of freshly prepared streptozotocin (STZ, dissolved in 10 mM citrate buffer, pH 4.2) at 40 mg/kg for five consecutive days. Two weeks after STZ injection, the mice were randomly separated into four groups and treated respectively with vehicle (V) (i.p. injection three times per week), paricalcitol (P) at 0.4 μg/kg (dissolved in propylene glycol:ethanol = 90:10, i.p. injection three times per week), losartan (L) (dissolved in drinking water at 0.025 mg/ml), or a combination of losartan and paricalcitol (L+P). Control (C) mice were nondiabetic mice without STZ or any

drug treatment. Blood glucose levels were monitored with the CONTOUR blood glucose monitoring system (Bayer) using one drop of tail blood. Spot urine was collected at various time points, and urinary albumin and creatinine levels were determined by using commercial kits as reported previously (25). All mice were killed at 13 weeks after the start of STZ treatment, and kidneys were immediately harvested for protein or RNA extraction or for histological analyses. The animal study protocol was approved by the Institutional Animal Care and Use Committee at the University of Chicago.

Western Blot. Western blot analyses were performed as described previously (54). Antibodies against FN and β-actin were from Sigma, antibody against Ang I/Ang II was from Santa Cruz Biotechnology, and anti-renin antibody was described previously (24).

Real-Time RT-PCR. First-strand cDNAs were synthesized from 4 μg of total RNAs in a 20-μl reaction using MML-V reverse transcriptase (Invitrogen) and hexanucleotide random primers. Real-time PCR was performed in an Applied Biosystems 7900 Real Time PCR System as described (25). The relative mRNA amount was normalized to the β2 microglobulin mRNA. The PCR primers used in this study are listed in Table S2.

Kidney Histology and Immunohistochemistry. Freshly dissected kidneys were fixed overnight with 4% formaldehyde in PBS (pH 7.2), processed, embedded in paraffin, and cut into 4-μm sections. Renal sections were stained with H&E or with periodic acid-Schiff (PAS). Semiquantitative scoring of glomerular sclerosis in PAS-stained slides was performed by using a five-grade method described previously (25, 55). Immunostaining of the sections was performed as described previously (25) with FN- and Ang II-specific antibodies.

Electron Microscopy. Kidney cortex was fixed in 2% glutaraldehyde and post-fixed in osmium tetroxide in 1% in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature. Electron microscopic analyses of the kidney and measurement of the GBM thickness were performed as described previously (25).

Statistical Analysis. Data values are presented as means ± SEM. Statistical comparisons were made by using Student's *t* test, with $P < 0.05$ being considered significant.

ACKNOWLEDGMENTS. We thank Anthony Chang for assistance in pathological evaluation of the kidney sections. This work was supported by National Institutes of Health Grants R21DK073183 and R01HL085793 and American Diabetes Association Grant 7-05-RA-80 (to Y.C.L.).

1. Ritz E, Rychlik I, Locatelli F, Halimi S (1999) End-stage renal failure in type 2 diabetes: A medical catastrophe of worldwide dimensions. *Am J Kidney Dis* 34:795–808.
2. Chan JC, *et al.* (2000) Long-term effects of angiotensin-converting enzyme inhibition and metabolic control in hypertensive type 2 diabetic patients. *Kidney Int* 57:590–600.
3. Lewis EJ, *et al.* (2001) Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes. *N Engl J Med* 345:851–860.
4. Brenner BM, *et al.* (2001) Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *N Engl J Med* 345:861–869.
5. Parving HH, *et al.* (2001) The effect of irbesartan on the development of diabetic nephropathy in patients with type 2 diabetes. *N Engl J Med* 345:870–878.
6. Andersen S, *et al.* (2000) Renoprotective effects of angiotensin II receptor blockade in type 1 diabetic patients with diabetic nephropathy. *Kidney Int* 57:601–606.
7. Price DA, *et al.* (1999) The paradox of the low-renin state in diabetic nephropathy. *J Am Soc Nephrol* 10:2382–2391.
8. Nishiyama A, Seth DM, Navar LG (2002) Renal interstitial fluid concentrations of angiotensins I and II in anesthetized rats. *Hypertension* 39:129–134.
9. Navar LG, *et al.* (1996) Paracrine regulation of the renal microcirculation. *Physiol Rev* 76:425–536.
10. Nguyen G, *et al.* (2002) Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. *J Clin Invest* 109:1417–1427.
11. Lavoie JL, Sigmund CD (2003) Minireview: Overview of the renin-angiotensin system—an endocrine and paracrine system. *Endocrinology* 144:2179–2183.
12. Anderson S, Jung FF, Ingelfinger JR (1993) Renal renin-angiotensin system in diabetes: Functional, immunohistochemical, and molecular biological correlations. *Am J Physiol* 265:F477–F486.
13. Zimpelmann J, *et al.* (2000) Early diabetes mellitus stimulates proximal tubule renin mRNA expression in the rat. *Kidney Int* 58:2320–2330.
14. Singh R, Singh AK, Alavi N, Leehey DJ (2003) Mechanism of increased angiotensin II levels in glomerular mesangial cells cultured in high glucose. *J Am Soc Nephrol* 14:873–880.
15. Vidotti DB, *et al.* (2004) High glucose concentration stimulates intracellular renin activity and angiotensin II generation in rat mesangial cells. *Am J Physiol* 286:F1039–F1045.
16. Yoo TH, *et al.* (2007) Activation of the renin-angiotensin system within podocytes in diabetes. *Kidney Int* 71:1019–1027.
17. Shankland SJ (2006) The podocyte's response to injury: Role in proteinuria and glomerulosclerosis. *Kidney Int* 69:2131–2147.
18. Gilbert RE, Krum H, Wilkinson-Berka J, Kelly DJ (2003) The renin-angiotensin system and the long-term complications of diabetes: Pathophysiological and therapeutic considerations. *Diabet Med* 20:607–621.
19. Carey RM, Siragy HM (2003) The intrarenal renin-angiotensin system and diabetic nephropathy. *Trends Endocrinol Metab* 14:274–281.
20. Li YC, *et al.* (2004) Vitamin D: A negative endocrine regulator of the renin-angiotensin system and blood pressure. *J Steroid Biochem Mol Biol* 89–90:387–392.
21. Yuan W, *et al.* (2007) 1,25-Dihydroxyvitamin D3 suppresses renin gene transcription by blocking the activity of the cyclic AMP response element in the renin gene promoter. *J Biol Chem* 282:29821–29830.
22. Xiang W, *et al.* (2005) Cardiac hypertrophy in vitamin D receptor knockout mice: Role of the systemic and cardiac renin-angiotensin systems. *Am J Physiol* 288:E125–E132.
23. Kong J, Li YC (2003) Effect of angiotensin II type I receptor antagonist and angiotensin-converting enzyme inhibitor on vitamin D receptor null mice. *Am J Physiol* 285:R255–R261.
24. Li YC, *et al.* (2002) 1,25-Dihydroxyvitamin D(3) is a negative endocrine regulator of the renin-angiotensin system. *J Clin Invest* 110:229–238.
25. Zhang Z, *et al.* (2008) Renoprotective role of the vitamin D receptor in diabetic nephropathy. *Kidney Int* 73:163–171.
26. Muller DN, Luft FC (2006) Direct renin inhibition with aliskiren in hypertension and target organ damage. *Clin J Am Soc Nephrol* 1:221–228.
27. Borghi C, *et al.* (1993) Evidence of a partial escape of renin-angiotensin-aldosterone blockade in patients with acute myocardial infarction treated with ACE inhibitors. *J Clin Pharmacol* 33:40–45.
28. Roig E, *et al.* (2000) Clinical implications of increased plasma angiotensin II despite ACE inhibitor therapy in patients with congestive heart failure. *Eur Heart J* 21:53–57.
29. Veniant M, *et al.* (1996) Vascular damage without hypertension in transgenic rats expressing prorenin exclusively in the liver. *J Clin Invest* 98:1966–1970.
30. Qiao G, Kong J, Uskokovic M, Li YC (2005) Analogs of 1alpha,25-dihydroxyvitamin D3 as novel inhibitors of renin biosynthesis. *J Steroid Biochem Mol Biol* 96:59–66.
31. Fryer RM, *et al.* (2007) Differential inhibition of renin mRNA expression by paricalcitol and calcitriol in C57/BL6 mice. *Nephron Physiol* 106:76–81.
32. Li YC (2007) Inhibition of renin: An updated review of the development of renin inhibitors. *Curr Opin Investig Drugs* 8:750–757.
33. Qi Z, *et al.* (2005) Characterization of susceptibility of inbred mouse strains to diabetic nephropathy. *Diabetes* 54:2628–2637.
34. Breyer MD, *et al.* (2005) Mouse models of diabetic nephropathy. *J Am Soc Nephrol* 16:27–45.
35. Kawachi H, *et al.* (2006) Role of podocyte slit diaphragm as a filtration barrier. *Nephrology (Carlton, Vic)* 11:274–281.
36. Liebau MC, *et al.* (2006) Functional expression of the renin-angiotensin system in human podocytes. *Am J Physiol* 290:F710–F719.
37. Gross JL, *et al.* (2005) Diabetic nephropathy: Diagnosis, prevention, and treatment. *Diabetes Care* 28:164–176.
38. Eijkelkamp WB, *et al.* (2007) Albuminuria is a target for renoprotective therapy independent from blood pressure in patients with type 2 diabetic nephropathy: Post hoc analysis from the Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan (RENAAL) trial. *J Am Soc Nephrol* 18:1540–1546.
39. Azizi M, *et al.* (2004) Pharmacologic demonstration of the synergistic effects of a combination of the renin inhibitor aliskiren and the AT1 receptor antagonist valsartan on the angiotensin II-renin feedback interruption. *J Am Soc Nephrol* 15:3126–3133.
40. Cooper ME (1998) Pathogenesis, prevention, and treatment of diabetic nephropathy. *Lancet* 352:213–219.
41. Perkins BA, *et al.* (2007) Microalbuminuria and the risk for early progressive renal function decline in type 1 diabetes. *J Am Soc Nephrol* 18:1353–1361.
42. Mogensen CE, Christensen CK (1984) Predicting diabetic nephropathy in insulin-dependent patients. *N Engl J Med* 311:89–93.
43. Viberti GC, *et al.* (1982) Microalbuminuria as a predictor of clinical nephropathy in insulin-dependent diabetes mellitus. *Lancet* 1:1430–1432.
44. Ficociello LH, *et al.* (2007) Determinants of progression from microalbuminuria to proteinuria in patients who have type 1 diabetes and are treated with angiotensin-converting enzyme inhibitors. *Clin J Am Soc Nephrol* 2:461–469.
45. Kuhlmann A, *et al.* (2004) 1,25-Dihydroxyvitamin D3 decreases podocyte loss and podocyte hypertrophy in the subtotally nephrectomized rat. *Am J Physiol* 286:F526–F533.
46. Agarwal R, *et al.* (2005) Antiproteinuric effect of oral paricalcitol in chronic kidney disease. *Kidney Int* 68:2823–2828.
47. Bonnet F, *et al.* (2001) Irbesartan normalises the deficiency in glomerular nephrin expression in a model of diabetes and hypertension. *Diabetologia* 44:874–877.
48. Benigni A, *et al.* (2001) Blocking angiotensin II synthesis/activity preserves glomerular nephrin in rats with severe nephrosis. *J Am Soc Nephrol* 12:941–948.
49. Takano Y, *et al.* (2007) Recovery and maintenance of nephrin expression in cultured podocytes and identification of HGF as a repressor of nephrin. *Am J Physiol* 292:F1573–F1582.
50. Zhang Z, *et al.* (2007) 1,25-Dihydroxyvitamin D(3) targeting of NF-kappaB suppresses high glucose-induced MCP-1 expression in mesangial cells. *Kidney Int* 72:193–201.
51. Kagami S, Border WA, Miller DE, Noble NA (1994) Angiotensin II stimulates extracellular matrix protein synthesis through induction of transforming growth factor-beta expression in rat glomerular mesangial cells. *J Clin Invest* 93:2431–2437.
52. Kaneshiro Y, *et al.* (2006) Increased expression of cyclooxygenase-2 in the renal cortex of human prorenin receptor gene-transgenic rats. *Kidney Int* 70:641–646.
53. Klaus G (2008) Renoprotection with vitamin D: Specific for diabetic nephropathy? *Kidney Int* 73:141–143.
54. Li YC, Bolt MJG, Cao L-P, Sitrin MD (2001) Effects of vitamin D receptor inactivation on the expression of calbindins and calcium metabolism. *Am J Physiol* 281:E558–E564.
55. Taneda S, *et al.* (2003) Amelioration of diabetic nephropathy in SPARC-null mice. *J Am Soc Nephrol* 14:968–980.